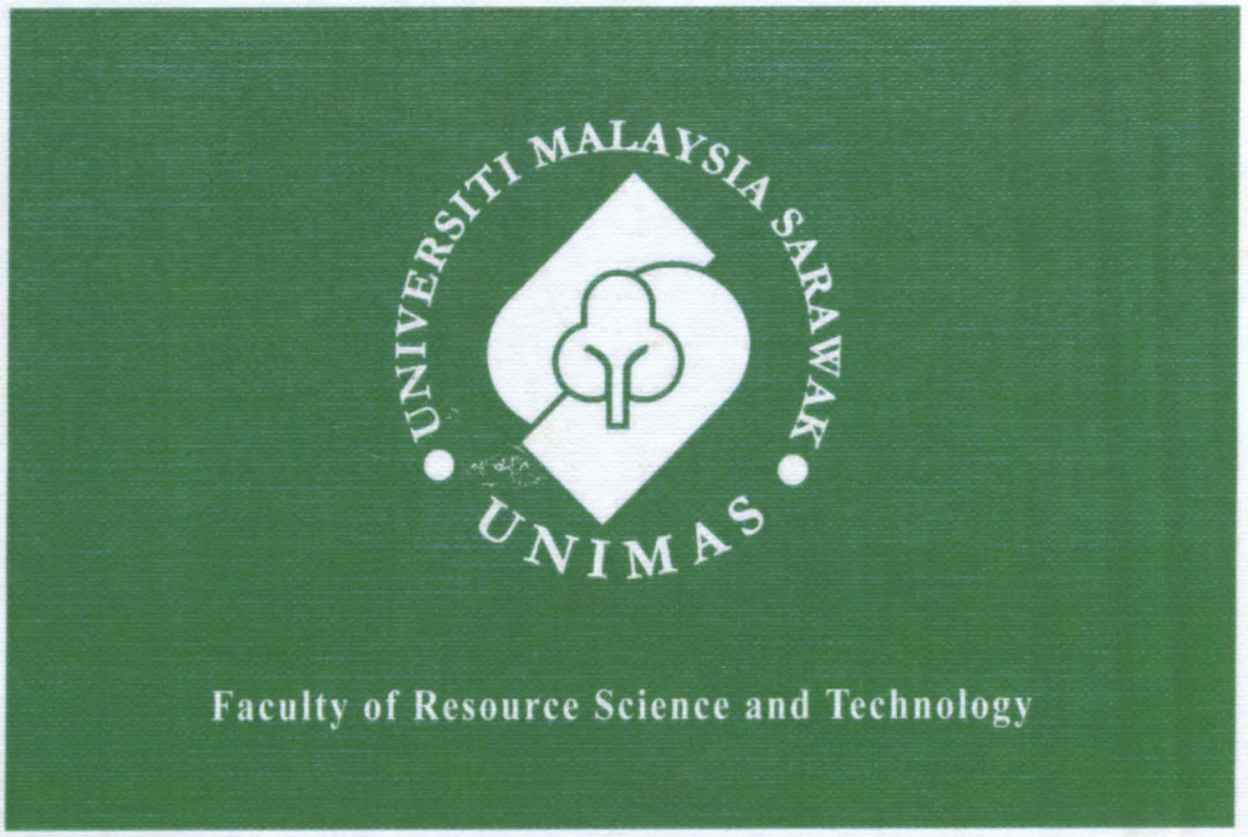

DEVELOPMENT OF SEROLOGICAL ASSAY FOR DETECTION OF CHIKUNGUNYA VIRUS INFECTION

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Bachelor of Science with Honours
(Resource Biotechnology)
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Development of serological assay for detection of Chikungunya virus infection

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A thesis submitted in fulfilment of the requirement for the degree of
Bachelor of Science with Honours

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UNIVERSITY MALAYSIA SARAWAK
2018

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Development of serological assay for detection of Chikungunya virus infection

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ABSTRACT

Chikungunya virus (CHIKV) is a mosquito-borne virus that infects human. The infection caused by CHIKV and dengue virus (DENV) share some common signs and symptoms which includes high fever, chills, rashes, joint and muscle pain. However, the prognosis, patient care, and persistent symptoms are different. The accurate identification of CHIKV is important during CHIKV outbreak, since dengue infection is endemic in Malaysia. To date, the diagnosis of CHIKV could only be done at Institute of Medical Research (IMR) and National Public Health Laboratory (NPHL) which are located at Peninsular Malaysia. Hence, the aim of this study is to develop a serological assay for the detection of CHIKV infection by the use of a recombinant antigen. The recombinant CHIKV E2 protein are expressed in *Escherichia coli (E. coli)* using pET SUMO expression system. The expressed protein was evaluated by SDS-PAGE and western blot. Then the E2 recombinant protein was purified through ProBond Purification System (Novex, CA, USA) and dialysed gradually in PBS. Then, ELISA are carried out by using the E2 recombinant protein against various serum and the sensitivity and specificity are 60.7% and 90% respectively.

Keywords: CHIKV, recombinant CHIKV E2 protein, ELISA, sensitivity, specificity.

ABSTRAK

Virus Chikungunya (CHIKV) adalah penyakit yang menjangkiti manusia melalui nyamuk. Jangkitan yang disebabkan oleh CHIKV dan denggi virus (DENV) berkongsi beberapa tanda biasa dan gejala termasuk demam, menggigil, ruam, sakit sendi dan otot. Walau bagaimanapun, prognosis, penjagaan pesakit, dan symptom berterusan adalah berbeza. Pengenalan tepat CHIKV adalah penting semasa wabak CHIKV, memandangkan jangkitan denggi adalah endemic di Malaysia,. Setakat ini, diagnosis jangkitan CHIKV hanya boleh dijalankan di Institut Penyelidikan Perubatan (IMR) dan Makmal Kesihatan Awam Kebangsaan (NPHL) yang terletak di Semenanjung Malaysia. Oleh itu, tujuan kajian ini adalah untuk membangunkan assay serologi untuk mengesan jangkitan CHIKV dengan menggunakan antigen rekombinan. Rekombinan protein CHIKV E2 telah diekspres di dalam Escherichia coli (E.coli) dengan menggunakan sistem ekspresi pET SUMO. Protein yang diekspres telah dinilai dengan menggunakan SDS-PAGE dan Western blot. E2 rekombinan protein telah dipurifikasi dengan menggunakan ProBond Purification System (Novex, CA, USA) dan dialysed dengan beransur-ansur dalam PBS . Kemudian, ELISA dijalankan dengan menggunakan protein rekombinan E2 terhadap pelbagai serum dan sensitivity dan spesifisiti adalah 60.7% dan 90% masing-masing.

Kata kunci: CHIKV, rekombinan CHIKV E2 protein, ELISA, sensitivity, spesifisiti

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List of Abbreviations

ARB	Arbidol
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
CHIK	Chikungunya
CHIKV	Chikungunya virus
Colony PCR	Colony polymerase chain reaction
DNA	Deoxyribose nucleic acid
ELISA	Enzyme linked immunosorbent assay
HPC	Hepatitis C virus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHCM	Institute of health and community medicine
IMR	Institute for Medical Research
IPTG	Isopropyl β-D-thiogalactoside
LB	Luria Bertani
NaCl	Sodium chloride
Nickel-HRP	Nickel with horseradish peroxidase
NPHL	National Public Health Laboratory

NSAIDs	Non-steroidal anti-inflammatory drugs
°C	Celcius
ORF	Open reading frame
PBS	Phosphate buffered saline
RNA	Ribose nucleic acid
R.O water	Reverse osmosis water
Rpm	Revolution per minute
RSV	Respiratory syncytial virus
RT-PCR	Real-time polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
S.O.C	Super optimal broth with catabolite repression
SFV	Semliki forest virus
TAE	Tris acetate EDTA
UHQ water	Ultra high-quality water

1.0 INTRODUCTION

Chikungunya virus (CHIKV) is an arthropod-borne virus with major outbreaks in Africa, India, and South-East Asia (Power and Logue, 2007). CHIKV are transmitted by the same species of mosquitoes *Aedes aegypti* and *Aedes ablopictus* (Thiboutot *et al.*, 2010). The major clinical symptom of CHIKV infection is febrile illness, which is clinically similar to symptoms of dengue virus infection (Karabatsos, 1975).

In Malaysia, molecular technique or immunofluorescence assay (IFA) are used for the detection of CHIKV infection. The molecular technique is used to detect the viral genome in the infected patient while IFA technique is used to detect the presence of antibodies against a specific antigen in serum. However, in Sarawak the validation of CHIKV infection can only be carried out through Institute for Medical Research (IMR) and National Public Health Laboratory (NPHL) at Peninsular Malaysia. Therefore, the identification of the infection will be delayed if an outbreak occurs because unlike dengue virus infection, CHIKV infection is rarely fatal and usually does not require close clinical supervision.

Due to the similar clinical symptoms of CHIKV infection with other virus infection such as dengue virus (DENV), CHIKV infection is often misdiagnosed (Morrison, 2014). Therefore, the ability to differentiate CHIKV infection from dengue virus infection would be important to have a proper control measures, particularly in areas where dengue virus infection is endemic or epidemic (Cho *et al.*, 2008). Hence, serological assay for the detection of CHIKV infection should be available in Sarawak. Thus, the aim of this study is to develop a serological assay for the detection of CHIKV infection.

Therefore, the objectives of this study are:

1. To express and purify the recombinant CHIKV E2 protein in *Escherichia coli* expression system.

2. To determine the specificity and sensitivity of serological assay developed for detection of CHIKV infection.

2.0 LITERATURE REVIEW

2.1 Chikungunya Virus

Chikungunya virus (CHIKV) belongs to the family *Togaviridae* and genus *Alphavirus*. The infection of CHIKV causes chikungunya fever (CHIK fever) which is a febrile illness linked with severe arthralgia and rash (McGill, 1995). The term 'Chikungunya' is derived from Makonde word meaning 'that which bends up' that refers to the contorted posture of the infected patients suffering from severe joint pain (Mavalankar *et al.*, 2008). CHIKV was first documented in 1952 during an epidemic outbreak in Makonde, Tanzania (Robinson, 1955). The transmission of CHIKV to humans are mostly through the bite of an infected *Aedes* mosquito. The transmission cycles of CHIKV includes urban cycle which is man – mosquito – man and sylvatic cycle animal – mosquito – man (Apandi *et al.*, 2009). The main vector that involves in urban cycles are usually *Aedes aegypti* and *Aedes albopictus* and *Aedes africanus*, *Aedes luteocephalus*, *Aedes furcifer* and *Aedes taylori* are the major vector involves in sylvatic cycle (Thiboutot *et al.*, 2010).

2.2 Genome of CHIKV

The genome of CHIKV (Figure 2.1) is a linear, single stranded, positive-sense RNA virus with approximately 11.8 kb and is capped in 5' and polyA tail in 3' end. The genome includes two open reading frames (ORFs) and encodes 4 non-structural proteins (nsP1, nsP2, nsP3 and nsP4) and five structural proteins (Capsid, E3, E2, 6k and E1) (Strauss *et al.*, 1991). The structural protein E1 and E2 associate as trimers of heterodimers (E2 – E1) as spikes on viral surface (Voss *et al.*, 2010) and the protein cover the viral surface in the form of membrane-anchored types (Cho *et al.*, 2008). The viral spikes help the attachment of the virus to cell surfaces and viral entry into the cells. The phylogenetic trees of CHIKV (Figure 2.2) shows that there are three

different genotypes based on geographical origins which are ECSA (East/Central/South African), West Africa, and Asian (Presti *et al.*, 2014). According to Schuffenecker *et al.* (2006), the single point mutation of alanine to valine at position 226 in E1 envelope glycoprotein (E1 – A226V1) during the Indian Ocean outbreak enhanced the ability of CHIKV replication in mosquitoes' vectors (Tsetsarkin *et al.*, 2007).

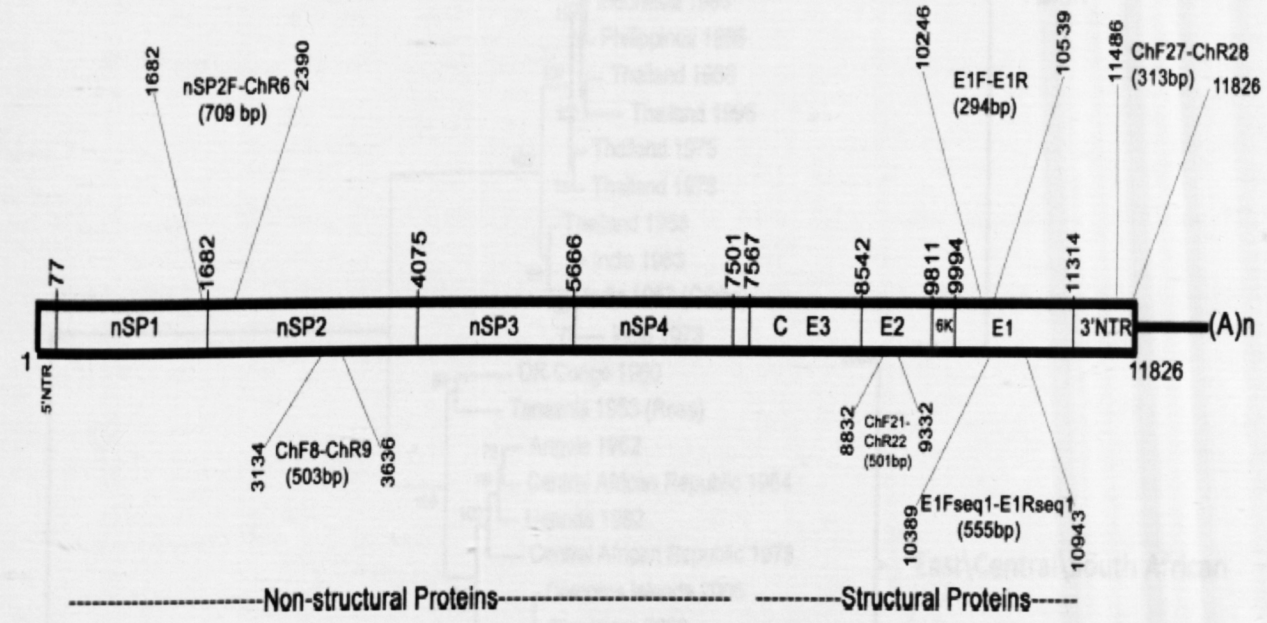

Figure 2.1 Genome of CHIKV (Niyas *et al.*, 2010).

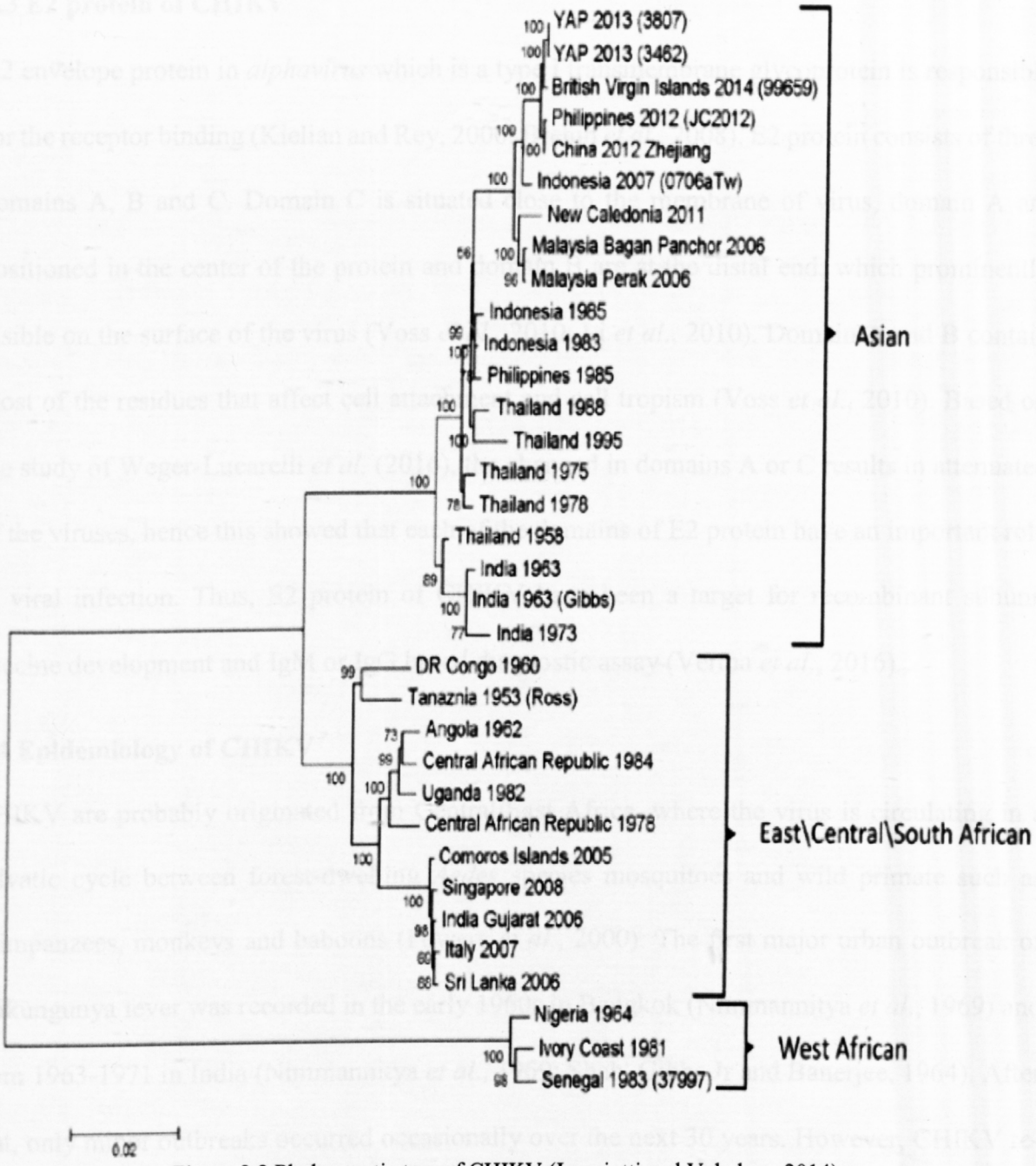

Figure 2.2 Phylogenetic tree of CHIKV (Lanciotti and Valadere, 2014)

2.3 E2 protein of CHIKV

E2 envelope protein in *alphavirus* which is a type I transmembrane glycoprotein is responsible for the receptor binding (Kielian and Rey, 2006; Brehin *et al.*, 2008). E2 protein consists of three domains A, B and C. Domain C is situated close to the membrane of virus, domain A are positioned in the center of the protein and domain B are at the distal end, which prominently visible on the surface of the virus (Voss *et al.*, 2010; Li *et al.*, 2010). Domain A and B contain most of the residues that affect cell attachment and cell tropism (Voss *et al.*, 2010). Based on the study of Weger-Lucarelli *et al.* (2016), the changed in domains A or C results in attenuated of the viruses, hence this showed that each of the domains of E2 protein have an important role in viral infection. Thus, E2 protein of CHIKV have been a target for recombinant subunit vaccine development and IgM or IgG based diagnostic assay (Verma *et al.*, 2016).

2.4 Epidemiology of CHIKV

CHIKV are probably originated from Central/East Africa, where the virus is circulating in a sylvatic cycle between forest-dwelling *Aedes* species mosquitoes and wild primate such as chimpanzees, monkeys and baboons (Powers *et al.*, 2000). The first major urban outbreak of chikungunya fever was recorded in the early 1960s in Bangkok (Nimmannitya *et al.*, 1969) and from 1963-1971 in India (Nimmannitya *et al.*, 1969; Shah, Gibbs Jr and Banerjee, 1964). After that, only minor outbreaks occurred occasionally over the next 30 years. However, CHIKV re-emerged during 2004 at Kenya and spread throughout countries around the Indian Ocean region, Southeast Asia and major epidemic outbreaks at La Réunion a French island from 2005 to 2006 (Renault *et al.*, 2007). During 2004 to 2007 the outbreaks happened continuously with hundreds of thousands of reported cases and novel geographical areas such as Europe (UK, Belgium, Germany, Czech Republic, Norway, Italy, Spain and France), Hong Kong, Canada, Taiwan, Sri

Lanka and USA (Powers and Logue, 2007). These reported cases in new geographical area (Figure 2.3) were directly associated with the return of tourists from India and island of Indian Ocean (Warner *et al.*, 2006).

The first CHIKV outbreak in Malaysia was documented during 1998-1999 at Port Klang which affected more than 51 persons (Lam *et al.*, 2001) followed by the outbreak in Bagan Panchor, Perak (Kumarasamy *et al.*, 2006), and Kinta district, Perak in 2006 (Noridah *et al.*, 2007). However, the third and largest outbreak was documented in Tangkak, Johor in 2008 (Apandi *et al.*, 2011).

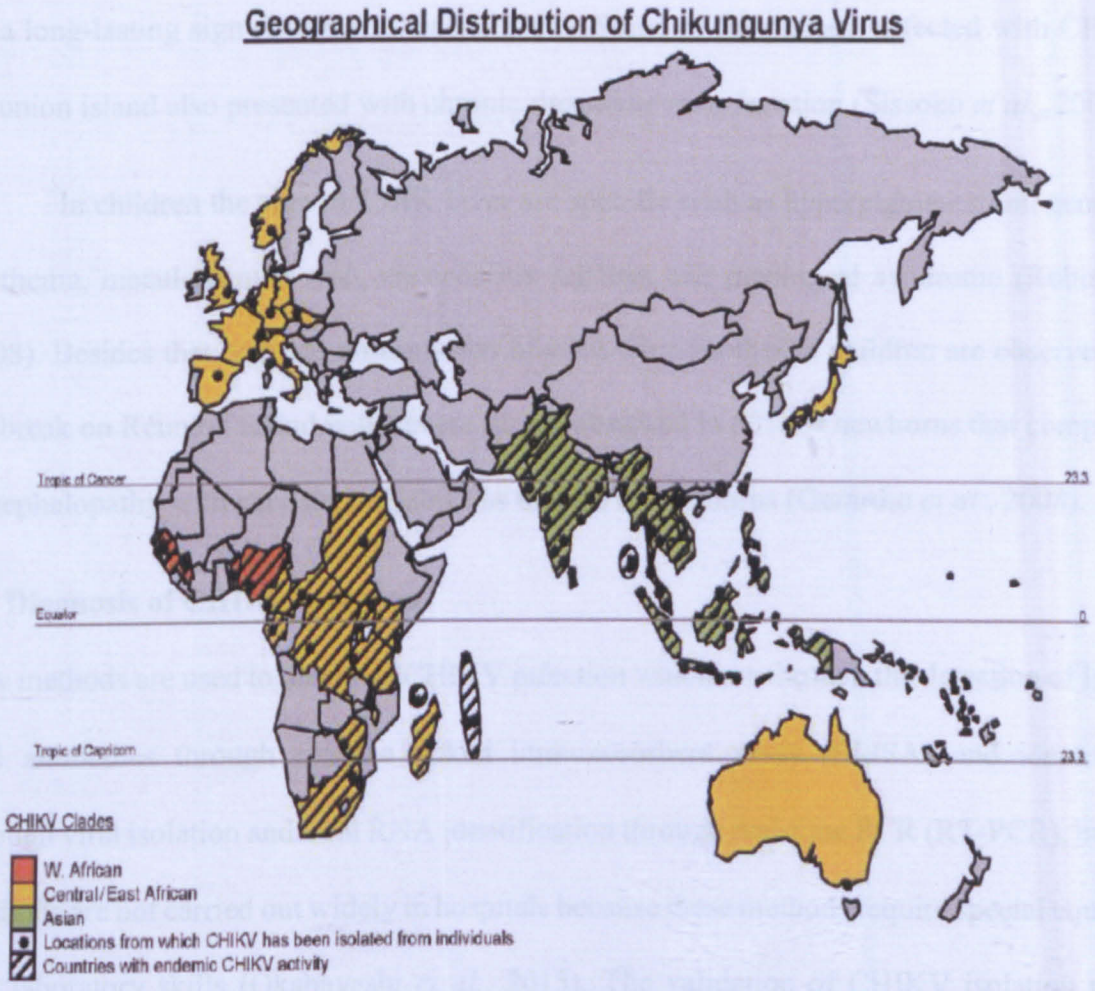

Figure 2.3 Worldwide distribution of CHIKV (Powers and Logue, 2007).

2.5 Clinical symptoms of CHIKV infection

The incubation period of CHIK fever is generally 2 – 10 days (Burt *et al.*, 2012; Singh and Unni, 2011). Some of the seroprevalence studies carried out and showed different rates of asymptomatic cases that ranges from 3.8% - 27.7% (Thiberville *et al.*, 2013). According to Win *et al.* (2010), during the acute stages of illness, patients with symptomatic cases typically shows the characteristics of high fever, polyarthralgia, headache, back pain and fatigue within 4 – 7 days of infection. The most distinctive symptom is polyarthralgia which is described in 87% - 98% of the symptomatic cases (Thiberville *et al.*, 2013). After the acute stages, some of the patients' experience recur or consistent symptoms such as arthralgia or musculoskeletal pains as a long-lasting sign (Borgherini *et al.*, 2008). Some of the patients infected with CHIKV in Réunion island also presented with chronic rheumatic manifestation (Sissoko *et al.*, 2009).

In children the sign of CHIK fever are specific such as hyperpigmentation, generalized erythema, maculopapular rash, encephalitis seizures and meningeal syndrome (Robin *et al.*, 2008). Besides that, vertical transmission of virus from mother to children are observed at the outbreak on Réunion island with severe illness observed in 53% of newborns that comprises of encephalopathy with persistent disabilities in 44% of newborns (Gerardin *et al.*, 2008).

2.6 Diagnosis of CHIKV infection

Few methods are used to diagnose CHIKV infection which are through the detection of IgM and IgG antibodies through enzyme linked immunosorbent assay (ELISA) and identification through viral isolation and viral RNA identification through real-time PCR (RT-PCR), but these methods are not carried out widely in hospitals because these methods require special equipment and laboratory skills (Okabayashi *et al.*, 2015). The validation of CHIKV isolation may be carried out through immunofluorescence or RT-PCR. RT-PCR can deliver a quick and sensitive

diagnosis, but it is not widely performed in hospitals because it requires specialist equipment and laboratory skills (Okabayashi *et al.*, 2015). However, this method is important for early diagnosis of CHIKV infection especially newborns with meningoencephalitis and vesiculobullous dermatitis (da Cunha and Trinta, 2017). An anti-CHIKV IgM kit is used in evaluation of patient with suspected CHIKV (Mourya and Mishra, 2006), but the IgM detection kit is only limited to the acute stages of illness (days 1 to 5) (Blacksell *et al.*, 2011). Thus, the development of new antigen based diagnostic assay is essential for a fast and reliable clinical diagnosis (Okabayashi *et al.*, 2015).

2.7 Treatment of CHIKV

Currently there are no specific treatment for CHIKV induced disease, and the treatment plans involve only supportive and symptomatic care (Goupil and Mores, 2016). The treatment for acute stages of infection consists of rest analgescis and antipyretics which includes the administration of non-steroidal anti-inflammatory drugs (NSAIDs) and chloroquine in case of refractory arthralgia (Simon *et al.*, 2007; Alla and Combe, 2011). NSAIDs are usually used to treat inflammation but administration of high doses of NSAIDs can control arthralgia but could cause thrombocytopenia, gastrointestinal bleeding, vomiting, gastritis and nausea (Pialoux *et al.*, 2007).

Some small molecule drugs that target the viral replication are still in experimental stages such as ribavirin, chloroquine and Arbidol. Ribavirin shows antiviral activity against a various type of RNA virus especially viruses in the genus *Alphavirus*, such as Semliki forest virus (SFV) and CHIKV (Ho, Ng and Chu, 2010). Currently, ribavirin is used in combination with interferon-α to treat hepatitis C virus (HPC) infection and respiratory syncytial virus (RSV) infections (Crotty, Cameron and Andino, 2002). Next, chloroquine are weak bases that could

affect acid vesicles and could causes the dysfunction of some enzymes. Some of the virus enter their host cell through endocytosis in lysosomal compartment and the low pH disrupts the viral particle and releases the viral nucleic acid. Thus, chloroquine can impair the replication of viruses by interacting with endosome mediated viral entry (Savarino *et al.*, 2003). The application of chloroquine as a treatment for viruses was documented more than 40 years ago (Inglot, 1969). However, for *alphavirus* SFV, the study of Maheshwari, Srikantan and Bhartiya (1991), shows that chloroquine enhanced virus replication in mice. Besides, the antiviral drug Arbidol (ARB) that has developed by the Center for Drug Chemistry, Moscow is effective against respiratory virus such as influenza A and B viruses and respiratory syncytial virus (RSV) (Delogu et al., 2011; Brooks *et al.*, 2012). A study also shows that ARB was effective against CHIKV *in vitro* by interfering with the early stages of infection such as the virus attachment and entry by targeting the cellular membrane (Delogu *et al.*, 2011).

2.8 Serological test

Serological test is effective in diagnosing infectious diseases. Through the principle of reaction of antigen-antibody, it is used to detect serum antibodies or antigen (Kim, Hwang and Oh, 2016). Serology is the detection of increasing titers of antibodies between an acute and convalescent stage of infection or detection of IgM in primary infection. There are few serological techniques that could be used which includes, ELISA, agglutination, precipitation, complement-fixation, fluorescent antibodies or western blot (Martinaskova and Valentova, 2012).

3.0 MATERIALS AND METHODS

3.1 LB medium & agar plate preparation

Luria Bertani (LB) medium consists of 5 g tryptone (BIO 101, CA, USA), 2.5 g yeast extract (amresco, OH, USA), 5 g NaCl (R&M, U.K) are dissolved with R.O water and the volume was adjusted to 500 ml. Then the media were autoclaved at 121 °C, 15 psi for 20 minutes (Hirayama, Japan). After the LB medium has cooled down, 0.5 ml of 50 µg/ml kanamycin (Appendix A.1.3) were added in biosafety cabinet (Envair, U.K) into the medium and stored at 4 °C until used. Then LB agar plate consists of 5 g tryptone (BIO 101, CA, USA), 2.5 g yeast extract (amresco, OH, USA), 5 g NaCl (R&M, U.K), 7.5g agar were dissolved with R. O water and the volume was adjusted to 500 ml. Then the LB agar were autoclaved and 0.5 ml of 50 µg/ml kanamycin (Appendix A.1.3) were added after the agar was cooled down to around 50 °C. After that the LB agar was poured into 20 different plates.

3.2 Bacteria culture growth

The colonies of CHIKV E2 in *E. coli* labelled with BS1/pE2/Mach1/COL1, BS1/pE2/Mach1/COL, BS1/pE2/Mach1/COL5, BS1/pE2/Mach1/COL6 and BS1/pE2/Mach1/COL7 together with the vector DNA colony pET SUMO/Mach1/COL4 were streaked on Luria Bertani (LB) agar plate containing 50 µg/ml kanamycin in a biosafety cabinet (Envair, U.K). The plates were incubated overnight at 37 °C in an incubator (Jisico, Korea).

3.3 Colony polymerase chain reaction (PCR)

First, colony PCR was done to check the presence of the E2 protein in the colonies. An individual colony was picked from plates by using a sterile toothpick and mixed into a 50 µl PCR reagent mixture that contain (5 µl 10X Taq buffer, 3 µl 25 mM $MgCl_2$, 1.5 µl pE2_F1 primer (20 pmol/µl), 1.5 µl pET SUMO reverse primer (10 pmol/µl), 1 µl 10 mM dNTP

(Fermentas, NY, USA), 0.5 µl Taq polymerase (Fermentas, NY, USA) and 37.5 µl UHQ water). The primer sequences used were shown in Table 3.1. For the pET SUMO control, the colony was picked and mixed in a 50 µl PCR reagent mixture that contain (5 µl 10X Taq buffer, 3 µl 25 mM $MgCl_2$, 1.5 µl pET SUMO forward primer (10 pmol/µl), 1.5 µl pET SUMO reverse primer (10 pmol/µl), 1 µl 10mM dNTP (Fermentas, NY, USA), 0.5 µl Taq polymerase (Fermentas, NY, USA), 37.5 µl UHQ water). The PCR was then performed in a thermocycler (PTC-200, MJ Research, NY, USA) in the condition shown in Table 3.2. Then, 1.5% agarose gel electrophoresis was run at 120 V for 40 minutes (Thermo Scientific, NH, USA) and the image was captured (Bio-Rad, CA, USA).

Table 3.1 Primers for colony PCR

Primers	Primer sequences
pET SUMO Forward	5'- AGA TTC TTG TAC GAC GGT ATT AG – 3'
pET SUMO Reverse	5'- TAG TTA TTG CTC AGC GGT GG – 3'

Table 3.2 Condition for colony PCR reaction

Condition	Temperature (°C)	Duration	Cycles
Initial denaturation	94	8 minutes	1
Denaturation	94	20 seconds	35
Annealing	55	30 seconds	
Extension	72	2 minutes	
Final extension	72	5 minutes	1